

ALPHA ARTEETHER RESISTANCE DOMAIN

Field of the Present Invention

The invention relates to a sequence of nucleotides in the DNA of *Escherichia coli* gyrase A gene which when changed provides resistance to α -arteether to the bacteria. This sequence can also be defined as the α -arteether resistance domain and can have wide spread application in the detection of resistance to α -arteether and related sesquiterpene endoperoxides. This invention also relates to the functional domain of gyrase A protein and in future can be utilized in structure/target based drug designing.

Background and prior art references of the invention

Since, ancient times, the plant *Artemisia annua* (family : Asteraceae) has been used as a traditional Chinese herbal medicine known as Qinghao for treating fever and malaria. The effective constituent was isolated by the Chinese investigators in 1972 and shown to be the sesquiterpene lactone, named artemisinin or qinghaosu (Jing-Ming et al., 1979; Tu et al., 1981). The structure of artemisinin has now been confirmed by a variety of analytical methods. Because artemisinin possesses high plasmodicidal property, it holds considerable promise for the treatment of drug resistant malaria. Artemisinin is poorly soluble in water and oil but readily soluble in most aprotic solvents. This property coupled with its short half-life, as well as desire to improve more potent derivatives, led to the efforts for chemically modifying its structure and synthesizing new artemisinin derivatives. Thus, ether derivatives of artemisinin called arteethers were prepared from dihydroartemisinin by etherification with ethanol in the presence of Lewis acid.

Absolute stereochemistry of arteethers (α and β isomers) at C-12 was also determined and it is 2-3 times more potent than artemisinin. The compounds α and β arteethers were developed as an antimalarial drug in India by the Central Institute of Medicinal and Aromatic Plants (CIMAP) and Central Drug Research Institute (CDRI) after phase III clinical trials. The arteether is presently sold in the market under the trade name E-MAL (injection).

In our earlier invention, we found a novel selective property of the compound α -arteether, which is inhibitory against the gyr mutant strains of *E. coli*, but ineffective against wild type strains (US Patent 6,127,405). Further we also developed in a separate patent 6,423,741 a strategic and novel combinations of a arteether which can be used as advanced generation

drug(s) to counter the resistance development itself while, having a potential to be used in treating infectious diseases particularly in those cases where drug resistant strains are known to appear very frequently.

The uniqueness and most useful feature is that, in a combination of a arteether and quinolone drugs and likewise, the spontaneous mutants arising resistant to quinolones or the derivatives will be killed by a arteether and at the same time any a arteether resistant strains become highly sensitive to nalidixic acid and hence eliminated by it through the combination approach. The new composition of compounds inhibits the resistance development due to mutation in the gyr A gene of bacteria, in which one component is a arteether and the other may be nalidixic acid or any of the fluoroquinolones (comprising of Ciprofloxacin, Norfloxacin, Levofloxacin, Sparfloxacin, Oxofloxacin and Lomefloxacin etc.) or compounds of similar nature against which the resistance may develop through a related process.

These above inventions were based on in vivo assays and do not indicate any insight into the genome for a arteether resistance mechanism based on which similar molecules can be structured/designed to target the DNA gyrase enzyme. Person skilled in the art may obtain clues from the present invention to design such molecules for a wide spectrum of microbes and hence the commercial importance of the invention.

Objects of the present Invention

The main object of the present invention is to identify an alpha-arteether resistance domain.

Another main object of the present invention is to develop a method of identifying an alpha-arteether resistance domain in a pathogen.

Summary of the present Invention

The invention relates to a sequence of nucleotides in the DNA of Escherichia coli gyrase A gene which when changed provides resistance to cc-arteether to the bacteria. This sequence can also be defined as the a-arteether resistance domain and can have wide spread application in the detection of resistance to a-arteether and related seaui terpene endoperoxides. This invention also relates to the functional domain of gyrase A protein and in future can be utilized in structure/target based drug designing.

Detailed description

Accordingly, the present invention relates to identifying an alpha-arteether resistance domain, and also, to developing a method of identifying an alpha-arteether resistance domain in a pathogen and thereby help in developing drugs against the pathogens.

In main embodiment of the present invention, wherein an oligonucleotide as alpha-arteether resistance domain (ADR) GGTCACCTCGGCGGTCTATGAC of SEQ ID No. 1.

In still another embodiment of the present invention, wherein the domain is from 241 to 261 nucleotide position of *gyr A* gene from translation start site of *E. Coli*.

In still another embodiment of the present invention, wherein an oligopeptide Gly Asp Ser Ala Val Tyr Asp of SEQ ID No. 2, corresponding to an oligonucleotide as alpha-arteether resistance domain (ADR) of SEQ ID No. 1.

In still another embodiment of the present invention, wherein the oligopeptide is from amino acid position 81 to 87 in *gyrase A* peptide of the enzyme.

In still another embodiment of the present invention, wherein a method of identifying alpha-arteether resistance domain (ADR) in a alpha-arteether resistant pathogens, to help develop drugs against the pathogen, said method comprising steps of:

- developing alpha-arteether resistant mutant from arteether sensitive strain,
- identifying both phenotypic and genotypic characteristics of the developed alpha-arteether resistant mutant, and
- identifying alpha-arteether resistance domain (ADR) in an alpha-arteether resistant pathogens.

In still another embodiment of the present invention, wherein an alpha-arteether resistance domain (ADR) is an oligonucleotide of SEQ ID No. 1

In still another embodiment of the present invention, wherein the ADR is from 241 to 261 nucleotide position of *gyr A* gene from translation start site of *E. Coli*.

In still another embodiment of the present invention, wherein the ADR has corresponding an oligopeptide of SEQ ID No. 2.

In still another embodiment of the present invention, wherein the oligopeptide is from amino acid position 81 to 87 in *gyrase A* peptide of the enzyme.

In still another embodiment of the present invention, wherein a set of three pairs of primers of SEQ ID Nos. 3, 4; 5, 6; and 7, 8.

In still another embodiment of the present invention, wherein the primers of SEQ ID Nos 3, 5, and 7 are forward primers.

In still another embodiment of the present invention, wherein the primers of SEQ ID Nos 4, 6, and 8 are reverse primers.

The next millenium will visualize a serious problem of antibiotic resistance for the antibiotics developed during the previous century and this is classified as a serious threat in the WHO report on infectious diseases. Currently antibiotics provide the main basis of causative therapy of bacterial infections. However high genetic variability of bacteria enable them to rapidly evade the action of antibiotics by developing resistance. Thus there has been a continuous search for new and potent antibiotics. The group of fluoroquinolones thus are vulnerable to this phenomena and our emphasis is to search new antibiotic from plant sources. In this context we have invented the semisynthetic compound ct-arteether and its effect on fluoroquinolone resistant bacteria (US Patent 6,127,405). The patent (US Patent 6,127,405) deals with the resistance developed against quinolone drugs and use of a-arteether to control the infections, but does not speak about the resistance development against

a-artether itself. The inventors studied this aspect of resistance development and experimentally proved that this cross-resistance (quinolone vs artether) development can be taken care of using the Combination therapy, which is not invented in the earlier invention (Patent No. 6,423,741). As the invention of a-artether as a agent to kill the quinolone resistant bacteria is new, no one knows about the resistance developing from a-artether. This led us to the invention of the drug resistance prevention system using a-artether and a fluoroquinolone drug. We studied at the DNA level to determine the mutations developed in the gyrase A gene of bacteria *Escherichia coli* (Kumar, S. 1976. *Journal of Bacteriology*, 125: 545-555.). This we assumed as the bacteria developed resistance against fluoroquinolones are sensitive to a-artether and the bacteria resistant to a-artether are resistant to fluoroquinolones. The fluoroquinolone drugs induce mutation in the gyrase A gene of bacteria and thereby the resistance developed.

There are consistent attempts to determine the mechanism of action of the antibiotics and based on which the newer antibiotics are designed by chemical modification of the prototype compounds. Simultaneously, attempts are being made to follow genomic approaches using the genomic database and identifying lethal targets. So, new drugs of fluoroquinolones group were developed to target bacterial type II topoisomerases which are otherwise known as DNA gyrases. Topoisomerases play an essential role for the control of the three-dimensional DNA structure in all cells. Among all topoisomerases bacterial type II topoisomerase (DNA gyrase) is unique by its ability to introduce the negative supercoils into covalently closed circular double stranded DNA in the relaxed state. DNA gyrase enzyme has two sub-units A and B. The quinolones and fluoroquinolones are targeted to the A subunit of the enzyme and shows bactericidal activity against dividing cells. This is due to the inhibition of the replicative DNA synthesis rather than the protein or RNA synthesis.

In our experiments planned to detect the biological activity of artethers against various strains of *E.coli*, we found an interesting feature that among the two isomers, only a isomer of artether is able to inhibit the growth of a particular *E.coli* strain DH5a available commercially, which carries a well defined mutation (gyrA 96) in the gene encoding DNA gyrase-A enzyme subunit. As a result of this mutation, the said strain is also resistant to a drug called nalidixic acid. The other *E.coli* strains which do not carry gyr mutation were invariably resistant to a-artether.

To ascertain the involvement of *gyrA* mutation, another *E. coli* strain NK 5819 which is also GyrA^r and nalidixic acid resistant was tested for arteether sensitivity. As expected, strain NK5819 was also sensitive only to a-isomer of arteether. For further substantiating the Gyr^r and a-arteether resistant relationship in *E. coli*, yet another strain MTCC 482 defective in DNA gyrase B subunit and hence termed GyrB^r was also tested for arteether sensitivity. Interestingly, even the *gyrB* MTCC 482 strain was susceptible only to a-isomer of arteether. The assays for arteether sensitivity was performed by the standard single disk diffusion method (Bauer et al, 1966). As a next step, we isolated *gyrA* mutants of *E. coli* strain CA 8000, which is otherwise Gyr⁺ and nalidixic acid sensitive. The *gyrA* mutants of CA8000 were isolated as nalidixic acid resistant colonies (20ug/ml), after mutagenic treatment with NTG (100 Hg/ml). The isolated CA8000 *gyrA* mutants were also sensitive only to a isomer of arteether. The above experiments clearly demonstrated the stereospecific inhibition of *gyr* mutants of *E. coli* by a-arteether. On the other hand, none of the above described *gyr* mutants were sensitive to the fl-isomer. To define precisely the involvement of *gyr* genes only in the a-arteether sensitivity, we utilised two recombinant clones (pMK 90 and pMK47 containing functional *gyr A* and *gyrB* genes respectively) in trans-complementation assays. For this purpose, we mobilized the plasmid clones into the *E. coli* *gyr* mutant strains. The resulting transformants were now nalidixic acid sensitive and a-arteether resistant. This constituted the direct evidence supporting our observation that DNA gyrase enzyme alone is involved in conferring a-arteether sensitivity to *E. coli* strain. Hence, the *gyr* strains of *E. coli* can be used as a biological sensor for detecting the a-isomer of arteether. DNA gyrase enzyme is essential for the bacterial growth. This enzyme transiently breaks the DNA strands and introduces negative superhelical turns in an ATP-dependent process. The *E. coli* DNA gyrase enzyme is a tetramer with two subunits A and B. These two subunits are nalidixic acid and coumermycin sensitive respectively (US Patent 6,127,405 and a co-pending patent).

The genes (*gyrA* and *gyrB*) encoding both these subunits have been isolated and cloned in *E. coli* and the prior arts define the drug resistance domain in *gyr A* gene for quinolones and fluoroquinolones. But the prior arts don't describe the resistance domains for a-arteether.

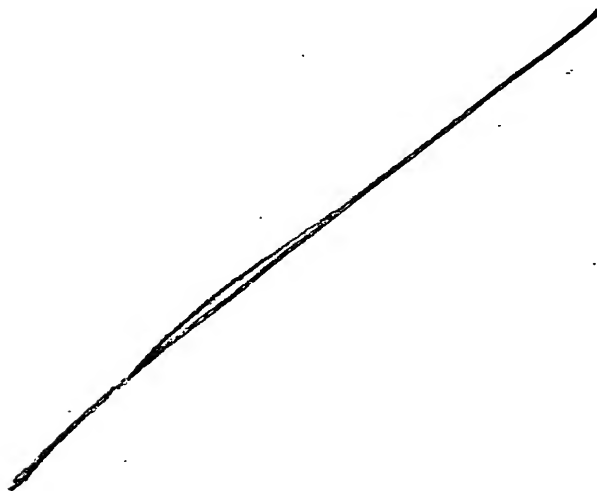
Quinolone Resistance Determining Region

Sequence analysis to DNA from many Bacterial species shows that resistance mutation tend to alter amino acid near the putative active site in the gyr A protein (Tyr 122 in E.coli). This region extending between amino acid 67 to 106 is called the QRDR . within gyr A of E.coli. Mutations of two codons Serine 83 to a hydrophobic amino acid generally confers more resistance than does mutation at position 87.

So in planned experiments we screened and isolated several gyr A spontaneous mutants resistant to a-artether from the a-artether sensitive strains. These a-artether sensitive strains were selected randomly from the quinolone and fluoroquinolone resistant strains. From the genebank database the gyr A gene sequence for Escherichia coli was downloaded and forward and reverse primers were designed and synthesized in ABI 392 DNA synthesizer.

The gyrase A gene of *Escherichia coli* and the primer sites.

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TGGCAAGACA AACGAGTATA TCAGGCATTG GATGTGAATA AAGCGTATAG -77
GTTTACCTCA AACTGCGCGG CTGTGTTATA ATTTGCGACC TTTGAATCCG -27
Forward -1          5' A ATTTGCGACC TTTGAATCCG 3'
                    +1
GGATACAGTA GAGGGATAGC GGTTAGATGA GCGACCTTGC GAGAGAAATT +24
ACACCGGTCA ACATTGAGGA AGAGCTGAAG AGCTCCTATC TGGATTATGC +74
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GATGTCGGTC ATTGTTGGCC GTGCGCTGCC AGATGTCCGA GATGGCCTGA +124
 AGCCGGTACA CCGTCGCGTA CTTTACGCCA TGAACGTACT AGGCAATGAC +174
 TGGAACAAAG CCTATAAAAA ATCTGCCCGT GTCGTTGGTG ACGTAATCGG +224
 TAAATACCAT CCCCATGGTG ACTCGGCGGT CTATGACACG ATTGTCCGCA +274
 TGGCGCAGCC ATTCTCGCTG CGTTATATGC TGGTAGACGG TCAGGGTAAC +324
 TTCGGTTCTA TCGACGGCGA CTCTGCGGCG GCAATGCGTT ATACGGAAAT +374
 CCGTCTGGCG AAAATTGCCC ATGAACTGAT GGCCGATCTC GAAAAAGAGA +424
 CGGTGCGATT CGTTGATAAC TATGACGGCA CGGAAAAAAT TCCGGACGTC +474
 ATGCCAACCA AAATTCCTAA CCTGCTGGTG AACGGTTCTT CCGGTATCGC +524
 CGTAGGTATG GCAACCAACA TCCCGCCGCA CAACCTGACG GAAGTCATCA +574
 ACGGTTGTCT GCGGTATATT GATGATGAAG ACATCAGCAT TGAAGGGCTG +624
 ATGGAACACA TCCCGGGGCC GGACTTCCCG ACGGCGGCAA TCATTAACGG +674
 TCGTCGCGGT ATTGAAGAAG CTTACCGTAC CGGTGCGGCG AAGGTGTATA +724
 TCCGCGCTCG CGCAGAAGTG GAAGTTGACG CCAAAACCGG TCGTGAAACC +774
 ATTATCGTCC ACGAAATTCC GTATCAGGTA AACAAAGCGC GCCTGATCGA +824
 GAAGATTGCG GAACTGGTAA AAGAAAAACG CGTGGAAGGC ATCAGCGCGC +874
 TGCGTGACGA GTCTGACAAA GACGGTATGC GCATCGTGAT TGAAGTGAAA +924
 CGCGATGCGG TCGGTGAAGT TGTGCTCAAC AACCTCTACT CCCAGACCCA +974
 Forward-2 5'ATGCGG TCGGTGAAGT TGTGCT3' 3G TTGGAGATGA GGGTCTGGGT
 GTTGCAGGTT TCTTTCGGTA TCAACATGGT GGCATTGCAC CATGGTCAGC +1024
 C'5 Reverse-1
 CGAAGATCAT GAACCTGAAA GACATCATCG CGGCGTTTGT TCGTCACCGC +1074
 CGTGAAAGTG TGACCCGTCG TACTATTTTC GAACTGCGTA AAGCTCGCGA +1124
 TCGTGCTCAT ATCCTTGAAG CATTAGCCGT GGCGCTGGCG AACATCGACC +1174
 CGATCATCGA ACTGATCCGT CATGCGCCGA CGCCTGCAGA AGCGAAAACT +1224
 GCGCTGGTTG CTAATCCGTG GCAGCTGGGC AACGTTGCCG CGATGCTCGA +1274
 ACGTGTGGC GACGATGTCG CGCGTCCGGA ATGGCTGGAG CCAGAGTTCCG +1324
 GCGTGCGTGA TGGTCTGTAC TACCTGACCG AACAGCAAGC TCAGGCGATT +1374
 CTGGATCTGC GTTTCAGAA ACTGACCGGT CTTGAGCACG AAAAAGTGTCT +1424
 CGACGAATAC AAAGAGCTGC TGGATCAGAT CGCGGAACGT TTGCGTATTC +1474
 TTGGTAGCGC CGATCGTCTG ATGGAAGTGA TCCGTGAAGA GCTGGAGCTG +1524
 GTTTCGTGAA AGTTTCGGTGA CAAACGTCGT ACTGAAATCA CCGCCAACAG +1574
 CGCAGACATC AACCTGGAAG ATCTGATCAC CCAGGAAGAT GTGGTCTGTA +1624
 CGCTCTCTCA CCAGGGCTAC GTTAAGTATC AGCCGCTTTC TGAATACGAA +1674
 GCGCAGCGTC GTGGCGGGAA AGGTAAATCT GCCGCACGTA TTAAAGAAGA +1724
 AGACTTTATC GACCGACTGC TGGTGGCGAA CACTCACGAC CATATTCTGT +1774
 GCTTCTCCAG CCGTGGTCGC GTCTATTCTGA TGAAAGTTTA TCAGTTGCCG +1824
 GAAGCCACTC GTGGCGCGCG CGGTGCTCCG ATCGTCAACC TGCTGCCGCT +1874
 Forward-3 5'TGCCGCT
 GGAGCAGGAC GAACGTATCA CTGCGATCCT GCCAGTGACC GAGTTTGAAG +1924
 GGAGCAGGAC GAA3' '3TAGGA CGGTCACTGG CTCAAAC'5 Reverse-2
 AAGGCGTGAA AGTCTTCATG GCGACCGCTA ACGGTACCGT GAAGAAAACT +1974
 GTCCTCACCG AGTTCAACCG TCTGCGTACC GCCGTAAG TGGCGATCAA +2024
 ACTGGTTGAC GGCGATGAGC TGATCGGCGT TGACCTGACC AGCGGCGAAG +2074
 ACGAAGTAAT GCTGTTCTCC GCTGAAGGTA AAGTGGTGCG CTTTAAAGAG +2124
 TCTTCTGTCC GTGCGATGGG CTGCAACACC ACCGTTGTTT CCGGTATTCCG +2174
 CTTAGGTGAA GCGGATAAAG TCGTCTCTCT GATCGTGCC TCGTGGCGATG +2224
 GCGCAATCCT CACCGCAACG CAAAACGGTT ACGGTAAACG TACCGCAGTG +2274
 GCGGAATACC CAACCAAGTC GCGTGCGACG AAAGGGGTTA TCTCCATCAA +2324
 GGTACCGAA CGTAACGGTT TAGTTGTTGG CGCGGTACAG GTAGATGACT +2374
 GCGACCAGAT CATGATGATC ACCGATGCCG GTACGCTGGT ACGTACTCGC +2424
 GTTTCGGAAT TCAGCATCGT GGGCCGTAAC ACCCAGGGCG TGATCCTCAT +2474
 CCGTACTGCG GAAGATGAAA ACGTAGTGGG TCTGCAACGT GTTGCTGAAC +2524
 CGGTGACGA GGAAGATCTG GATACCATCG ACGGCAGTGC CGCGGAAGGG +2574
 GACGATGAAA TCGCTCCGGA AGTGGACGTT GACGACGAGC CAGAAGAAGA +2624
 ATAATTTTAC TTCTTCATGC CAAAAGGGAG CTATCTCCCT TGTTTGAATT +2674
 '3TAGAGGGA ACAAACTTAA
 GAAAAGTCCA GGCTGCAAAG TCTGGGCTTT TGTCGTATTA GGGCACGGTA +2724
 CT'5 Reverse-3
 AAGTTTGGCT GTGCCCGTAA AAAATGGCTG GCTATACACA AGGAATGTGG +2774
 CAATGAGTGG TGAAAAAAG GCGAAAGGCT GGCGGTTCTA TGGTCTTGTA +2824
 GGTTTTGGCG CAATAGCACT GCTTTCGCT GGCGTCTGGG CGTTGCAATA +2874
 TGCTGGCAGT GGGCCAGAAA AAACGTTGTC GCCGCTGGTG GTGCACAACA +2924
 ATCTGCAAT CGATCT +2940

Size of the gyr A gene 2628 bases

Using the primer pair as mentioned below of SEQ ID No. 3 and 4 respectively, 1023 base pair sequence was amplified and sequenced (-48 to +975).

Forward-1 5'A ATTTGCGACC TTTGAATCCG 3' (21 bases)

Reverse-1 5'CTGGGTCTGGGAGTAGAGGTTG 3' (22 bases)

Using the primer pair SEQ ID No. 5 and 6 respectively as mentioned below 993 base pair sequence was amplified and sequenced (-929 to +1921).

Forward-2 5'ATGCGGTCGGTGAAGTTGTGCT 3' (22 bases)

Reverse-2 5'CAAAC TCGGTCACTGGCAGGAT 3' (22 bases)

Using the primer pair SEQ ID No. 7 and 8 respectively as mentioned below 809 base pair sequence was amplified and sequenced (+1868 to 2676).

Forward-2 5'TGCCGCTGGAGCAGGACGAA 3' (20 bases)

Reverse-2 5'TCAATTCAAACAAGGGAGAT 3' (20 bases)

Using these primers the total gyrase A gene was amplified to three fragments separately, purified and sequenced in ABI prism 377 automatic DNA sequencer. The sequences of the mutants were compared to the wild type.

Development of α -arteether resistant mutant from α -arteether sensitive mutants:

In order to develop an arteether resistant mutants from an arteether sensitive strains a reverse strategy was applied. Quinoline/fluoroquinolone (Q/FQs) resistant strains were developed spontaneously i.e. screening overnight log phase culture of the Quinolone / fluoroquinolone sensitive i.e. arteether resistant strain (*E coli* CA 8000) on the poison agar plate of the Q/FQs. The Q/FQ resistant mutants which appeared on the poison agar plates were first purified through single colony purification and then checked for their arteether sensitivity. These arteether sensitive mutants on the contrary when checked for their Q/Fqs resistance they show different level of Q/FQs resistance.

Bioactivity of beta-arteether on Selected Q/FQr mutants compared to *E. coli* CA 8000:

The strains of *E. coli* mutants were grown on Luria agar medium and beta-arteether (250 µg/ disc) was applied on disc. The clear zone around the disc killing the bacteria is called Zone of Inhibition (ZOI). This is the measure of sensitivity of the strain towards the compound alpha-arteether.

Table 1

Strains of <i>E. coli</i> / Mutants	Net ZOI in mm (250 µg / disc)
CA8000 (gyrA+)	-
DH5 α gyrA-	5
ET8000 (gyrA-)	5
NK5819 (gyrA-)	7
CA8001	10
CA8002	5
CA8003	9
CA8006	11
CA8007	8
CA8009	14
CA8010	10
CA8012	8

Level of Cross resistance of different mutants compared to *E. coli* CA 8000:

The minimum inhibitory concentrations (MIC) of the quinolone and fluoroquinolone antibiotics were quantified for different strains and compared among each other. The differential response indicate the measure of resistance and sensitivity. Higher MIC indicate more resistance.

Table 2

Mutant Strains	Nalidixic acid µg/ml	Norfloxacin µg/ml	Sparfloxacin µg/ml	Ciprofloxacin µg/ml	Lomefloxacin µg/ml
CA8000	2.0	<0.05	<0.05	<0.05	<0.05
DH5a	50	20	10	0.1	0.5
ET8000	100	50	20	0.5	>2.0
NK5819	20	25	10	<0.1	<0.5
CA8001	150	15	20	<0.1	1.5
CA8002	150	15	20	<0.1	1.5
CA8003	150	15	20	<0.1	2.5
CA8005	150	10	20	0.1	0.5
CA8006	150	20	40	0.1	1.5
CA8007	100	15	20	0.1	1.5
CA8009	100	15	40	0.1	1.5
CA80010	100	15	40	0.1	1.5
CA80012	100	15	40	0.1	1.5

Phenotypic as well as Genotypic characteristics of the mutants:

The strains of E coli of the investigation, their phenotypes in terms of a-arteether (resistance or sensitivity, superscript R or S) or antibiotic resistance or sensitivity, change in nucleotide position of the gyrase A gene leading to the phenotype, corresponding amino acid change in the protein and the position of amino acid change are being described. Always the phenotype a-arteether resistance is accompanied with quinolone or fluoroquinolone sensitivity and *vice versa*.

Table 3

S.No	E.coli Strains	Phenotype	Change in nucleotide (Position)	Change Aminoacid	in	Position of changed Aminoacid
1	DH5α	a-arteether ^S / Nal ^R	GAC -----AAC (259)	Asp-----Asn.		87
2	ET8000	a-arteether ^S / Nal ^R	GAC-----AAC(259)	Asp -----Asn.		87
3	NK5819	a-arteether ^S / Nal ^R	GAC-----TAC(259)	Asp -----Gly		87
4	CA8001	a-arteether ^S / Nal ^R Mutant	GGT-----TGT(241)	Gly-----Cys		81
5	CA8002	a-arteether ^S / Nal ^R Mutant	GGT-----TGT(241)	Gly-----Cys		81
6	CA8003	a-arteether ^S / Nal ^R Mutant	GAC-----GGC(260)	Asp-----Gly		87
8	CA8005	a-arteether ^S / Nal ^R Mutant	GAC-----GGC(260)	Asp-----Gly		87
9	CA8006	a-arteether ^S / Nal ^R Mutant	TCG-----TTG(248)	Ser-----Leu		83
10	CA8007	a-arteether ^S / Nal ^R Mutant	TCG-----TTG(248)	Ser-----Leu		83
12	CA8009	a-arteether ^S / Nal ^R Mutant	TCG-----TTG(248)	Ser-----Leu		83
13	CA8010	a-arteether ^S / Nal ^R Mutant	TCG-----TTG(248)	Ser-----Leu		83
15	CA8012	a-arteether ^S / Lome ^R Mutant	GGT-----GAT(242)	Gly-----Asp.		81

Phenotypic as well as genotypic characters (gyr A gene, QRDR region) of the WT as well as revertants :

The commercially available strain *E. coli* DH5 α (Stratagene, USA) is sensitive to \square -arteether but resistant to quinolone/ fluoroquinolone due to a mutation at 87th aminoacid position of the gyr A protein (Aspartic acid of wild type is changed to Asparagine) with corresponding change in the codon GAC to AAC. This strain was used to screen \square -arteether resistant mutations. The observation was startling as the \square -arteether resistant mutants are the exact reversion at the 87th position of the aminoacid of gyrase A subunit i.e. Asparagine changed to aspartic acid, with corresponding change in the codon AAC to GAC. Similarly When the mutants (a-arteether^S / Lom^R) were searched for a-arteether sensitivity those were found to be exact revertants at aminoacid position 81.

Table 4

S.No	<i>E.coli</i> Mutant Strains	Phenotype	Change in nucleotide	Change in Aminoacid	Position of changed Aminoacid
2	AR 9 CA8014	a-arteether ^R / Nal ^S Mutant	AAC-----GAC (259)	Asn-----Asp	87
3	Rev. 1	a-arteether ^R / Lom ^S Mutant	GAT-----GGT(242)	Asp-----Gly	81
4	Rev. 2	a-arteether ^R / Lom ^S Mutant	GAT-----GGT(242)	Asp-----Gly	81
5	Rev. 3	a-arteether ^R / Lom ^S Mutant	GAT-----GGT(242)	Asp-----Gly	81
6	Rev 4	a-arteether ^R / Lom ^S Mutant	GAT-----GGT(242)	Asp-----Gly	81
7	Rev 5	a-arteether ^R / Lom ^S Mutant	GAT-----GGT(242)	Asp-----Gly	81
8	Rev 6	a-arteether ^R / Lom ^S Mutant	GAT-----GGT(242)	Asp-----Gly	81

The α -arteether resistance domain(ARD) can be defined as the domain from 241 nucleotide position to 261 nucleotide position of *gyr A* gene from the translation start site (ATG codon) of *Escherichia coli* corresponding to 81 to 87 amino acid position in the gyrase A peptide of the enzyme from the N-terminal end. The N-terminal end can be defined as the starting amino acid in the peptide.

The ARD may extend further beyond the above mentioned nucleotide positions on either direction of the range mentioned and not limited to *Escherichia coli* only as the *gyr. A* gene is highly conserved among different bacterial species.

The commercially available strain *E.coli* DH5 α (Stratagene, USA) is sensitive to α -arteether but resistant to quinoline/fluoroquinolone due to a mutation at 87th aminoacid position of the *gyr A* protein (Aspartic acid of wild type is changed to Asparagine) with corresponding change in the codon GAC to AAC. This strain was used to screen α -arteether resistant mutations. The observation was starting as the α -arteether resistant mutants are the exact reversion at the 87th position of the amino acid of gyrase A subunit i.e. Asparagine changed to Aspartic acid, with corresponding change in the codon AAC to GAC.